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RESEARCH PAPER

Genes associated with opening and senescence of *Mirabilis jalapa* flowers

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Abstract

A modest ethylene climacteric accompanies flower senescence in *Mirabilis jalapa* L., and exogenous ethylene accelerates the process. However, inhibitors of ethylene action and synthesis have little effect on the life-span of these ephemeral flowers. Treatment with α-amanitin, an inhibitor of DNA-dependent RNA synthesis, substantially delays the onset of senescence. This effect falls linearly between 7 h and 8 h after the start of flower opening. Subtractive hybridization was used to isolate transcripts that were up- and down-regulated during this critical period. Eighty-two up-regulated and 65 down-regulated transcripts were isolated. The genes identified encode homologues of a range of transcription factors, and of proteins involved in protein turnover and degradation. Real-time quantitative RT-PCR was used to examine expression patterns of these genes during flower opening and senescence. Genes that were identified as being down-regulated during senescence showed a common pattern of very high expression during floral opening. These genes included a homologue of *CCA1*, a 'clock' gene identified in *Arabidopsis thaliana* and an aspartyl protease. Up-regulated genes commonly showed a pattern of increase during the critical period (4–9 h after opening), and some showed very strong up-regulation. For example, the abundance of transcripts encoding a RING zinc finger protein increased >40 000 fold during the critical period.

Key words: Circadian, ethylene, floral senescence, *Mirabilis jalapa*, senescence-associated genes, transcription factor, ubiquitin ligase.

Introduction

Three general patterns of floral senescence based on differences in how flowers respond to ethylene are recognized. In ethylene-dependent flowers, such as carnation, a rise in endogenous ethylene production triggers senescence (Woltering and van Doorn, 1988; van Doorn, 2001). If such flowers are treated with ethylene biosynthesis or action inhibitors, their life is extended substantially (Veen and van de Geijn, 1978; Fujino *et al*., 1980; Serek *et al*., 1994). Since ethylene is the endogenous senescence trigger, exogenous ethylene accelerates senescence (Woltering and van Doorn, 1988). In ethylene-independent flowers such as iris, there is little ethylene produced before or during senescence. Exogenous ethylene does not accelerate senescence and ethylene inhibitors do not elongate floral longevity (Woltering and van Doorn, 1988). Some flowers such as daffodil show an intermediate pattern of senescence. Without pollination, their senescence resembles that of the ethylene-independent flowers, in that there is little ethylene production and only a limited response to inhibition of ethylene biosynthesis and action. However, pollination results in an ethylene-dependent type of senescence, with an associated rise in endogenous ethylene production. Application of exogenous ethylene accelerates their senescence (Hunter *et al*., 2002).

Genes associated with senescence have been isolated from a number of flowers, including representatives with each of the three patterns of floral senescence (Lawton *et al*., 1990; Valpuesta *et al*., 1995; Rubinstein, 2000; Channelière *et al*., 2002; Hunter *et al*., 2002; van Doorn *et al*., 2003; Breeze *et al*., 2004). Early studies used differential screening of cDNA libraries, and demonstrated changes in expression of genes encoding catabolic enzymes such as proteases and nucleases, and in ethylene-dependent

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flowers, the key enzymes in ethylene biosynthesis, ACC synthase and ACC oxidase. Recently, PCR-based subtractive hybridization was successfully used in daffodil (Hunter *et al*., 2002), *Alstroemeria* (Breeze *et al*., 2004), and iris (van Doorn *et al*., 2003) to isolate larger populations of genes associated with floral senescence. Fifty-four genes were isolated from daffodil, including genes encoding a few regulatory proteins and several cysteine and serine proteases (Hunter *et al*., 2002). van Doorn *et al*. (2003) used a microarray approach to investigate changes in gene expression during development and senescence of iris flowers and identified a cluster of genes that were highly expressed during senescence. The 51 sequences in this cluster included a number of genes with unknown function, including a group with no significant homology to known sequences. They suggested that sequences encoding a Grap2 and cyclin-D interacting protein, a MADS-domain transcription factor, a casein kinase, and a nucleotide gated ion channel-interacting protein might be important elements in the regulation of senescence (van Doorn *et al*., 2003). Breeze *et al*. (2004) identified 93 up-regulated and 17 down-regulated genes associated with flower senescence in *Alstroemeria*. The up-regulated genes encoded diverse proteins, such as a zinc finger protein, an Xa21 receptor-type protein kinase, and an aspartic proteinase. Among the down-regulated genes were sequences encoding a gibberellin-induced protein and a cytochrome P450 (Breeze *et al*., 2004).

Although previous studies have demonstrated tantalizing changes in expression of genes that seem likely to play a role in the control of flower senescence, the function of these genes has not yet been demonstrated. The species chosen, particularly for studies of ethylene-insensitive senescence, such as daylily, iris, and daffodil, are difficult to transform and regenerate, and normally flower only after several years, so that determining the effect of up- or down-regulation of a single gene might take several to many years. Therefore an alternative model species was sought that showed intermediate senescence behaviour, had a short life cycle so that phenotyping would be relatively rapid, and was dicotyledonous, and therefore might more easily be transformed and regenerated.

Mirabilis jalapa (four o'clock, marvel of Peru) is a bushy perennial and has a very rapid seed to seed cycle – 10 weeks under ideal conditions. The flowers open in the early evening and collapse rapidly the next morning. Detached flowers (and even isolated corollas) have the same pattern of senescence as attached flowers, and a regeneration and transformation system has recently been reported (Zaccai *et al*., 2007). This species therefore provides an excellent model for investigating flower senescence.

In preliminary studies, it had been determined that the flowers of *M. jalapa* show a mixed senescence pattern. Although flower senescence in *M. jalapa* is accelerated by exogenous ethylene, inhibition of ethylene synthesis or action has little effect on flower longevity. Therefore changes in gene expression in this species were studied with a view to isolating and testing the function of potential regulatory genes.

Materials and methods

Plant materials and senescence observations

Mirabilis jalapa plants were grown in the greenhouse at the University of California, Davis under 16 h/8 h day/night cycles and 25/20 °C day/night temperature conditions. Flowers used in the physiological experiments were collected at the appropriate stages just before use. Flowers used in the subtractive hybridization were collected at the appropriate stages, and the isolated corollas were immediately frozen in liquid nitrogen and stored at −80 °C until needed. To determine the chronology of flower senescence and the effect of different inhibitors, time-lapse videography was used. Flowers or isolated corollas were placed in 1.5 ml Eppendorf tubes containing deionized water or a treatment solution. Time-lapse photographs were made with a digital video recorder connected to a computer running a commercial time-lapse capture program (DV Capture). Images were normally captured at 3 min intervals for the life of the flower. Eight readily identified stages of opening and senescence were established (Fig. 1) (Gookin *et al*., 2003), from small bud (stage 1, 24 h prior to opening), through the 7 stages (2–8) on the day of opening and senescence. Stage 3 (cracked bud) was considered the start of opening, and was used as a zero time point in chronological experiments (Gookin *et al*., 2003). The time taken to reach different stages was used to evaluate the effect of different treatments on opening and senescence.

Treatment with ethylene and 1-methylcyclopropene (1-MCP)

Harvested flowers or isolated corollas were placed in a small volume of water in a plastic rack and were sealed in a small transparent chamber. Ethylene was injected into the chamber to a final concentration of 2 μl l^{-1}. For 1-MCP treatments, flowers or corollas were treated with a concentration of 200 nl l^{-1} for 1 h as previously described (Serek *et al*., 1994).

Treatments with silver thiosulphate (STS) and aminooxyacetic acid (AOA)

Mirabilis jalapa flowers were collected in the long bud stage, about 2 h before opening. The corollas were detached and immediately placed in deionized water, 50 μM or 100 μM STS solution, or 1 mM or 2 mM AOA solution. The time taken for the corollas to progress from stage 3 (cracked bud) to stage 7 (first fold) was recorded.

Treatment with α-amanitin

Mirabilis jalapa flowers were labelled on the plant at stage 3 (cracked bud), then harvested at 5 min intervals from 7 h to 8.5 h after stage 3. Ten flowers were collected at each time point. After collection, the corollas were isolated and immediately placed in 20 μg ml^{-1} α-amanitin. Control flowers were placed in deionized water. The time taken for the corollas to progress from stage 3 to stage 7 (first fold) was recorded. When this time was at least 20% greater than in the control flowers, α-amanitin was considered to be having an inhibitory effect. The percentage of the corollas responding to α-amanitin was calculated for each harvest time.

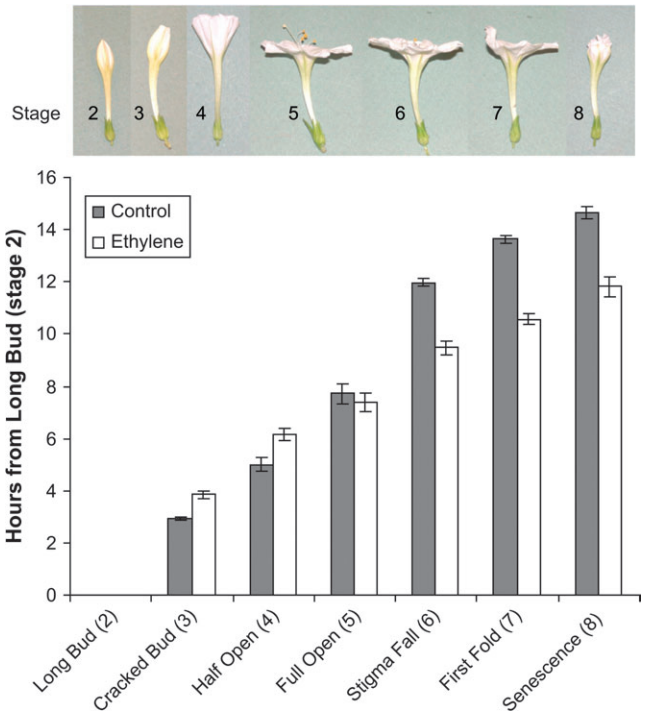

Fig. 1. Time course of development and effect of ethylene on the life of *M. jalapa* flowers. Flower opening and senescence was divided into eight stages, including small bud (stage 1, 24 h prior to opening), and the 7 stages shown (2–8). To test the effect of ethylene, flowers were harvested at the long bud stage, and sealed in transparent chambers containing air (control) or 2 μl l^{-1} ethylene. Time-lapse video images were recorded and used to assess the time taken to reach different stages during opening and senescence.

RNA isolation

Total RNA was extracted from a 1 g aliquot of frozen corolla tissue using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacture's instructions, and was treated with RNase-free DNase I (Promega, Madison, WI, USA) to remove any contaminating genomic DNA (Chen *et al*., 2004).

Subtractive library construction and differential screening

Subtractive libraries were constructed in both directions using RNA from corollas collected at 4 h and 9 h after opening. First strand cDNA was synthesized from 1 μg total RNA using the SMART cDNA Synthesis Kit (BD Biosciences, Mountain View, CA, USA). A PCR strategy was used to synthesize second-strand cDNA. To avoid over-amplification of cDNA the PCR reactions were optimized and conducted over 17 cycles in all samples. The PCR products were used to construct libraries using a PCR-Select cDNA Subtraction Kit (BD Biosciences) following the manufacturer's instructions. After confirmation of subtraction efficiency in both libraries, the subtracted cDNAs were cloned into pGEM-T Easy vectors (Promega) which were then used to transform *Escherichia coli* DH5α.

Differential screening of up-regulated and down-regulated libraries was performed using the PCR-Select Differential Screening Kit (BD Biosciences) following the manufacturer's instructions. Colony PCR was performed on 1026 colonies from the down-regulated library and 999 from the up-regulated library, following the manufacturer's instructions. The PCR products were arrayed on Hybond-N^+ nylon membranes (Amersham Biosciences, Piscataway, NJ, USA), then hybridized to probes synthesized from RNA extracted from 4 h and 9 h corollas. Hybridization was visualized on a Molecular Dynamics STORM® Phosphorimager System (Amersham Biosciences).

Plasmids containing differentially regulated sequences were isolated from their bacterial hosts using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The inserts were then sequenced at the Sequencing Facility on the UC Davis campus.

Sequence analysis

Sequences were assembled to tentative contigs using the SeqMan software (DNASTAR, Madison, WI, USA). Database searches were carried out using the BLAST program (NCBI). Alignments of multiple protein sequences were carried out using the ClustalX program.

Quantitative RT-PCR

First strand cDNA was synthesized from 2 μg of total RNA, oligo d(T) primer, random hexamers, and PowerScript reverse transcriptase (BD Biosciences). This cDNA was used as template for real-time PCR. The abundance of 18S rRNA was used as an internal control. Data were analysed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and are presented as the relative level of gene expression. The primers used in this study were designed based on the sequence information for each clone, using the Primer3 software, and are described in Table 1.

Results

Response to ethylene and ethylene inhibitors

Treatment with exogenous ethylene shortened flower life by approximately 20% (Fig. 1). Although there was no significant difference in flower opening (the time taken to reach stage 5), the ethylene-treated flowers showed a dramatic decrease in the time taken to progress from full open (stage 5) to stigma fall (stage 6). In control flowers, this change took about 4 h; in ethylene-treated flowers it only took 2 h (Fig. 1). This difference was maintained through final senescence.

The longevity of flowers treated with 1-MCP and held in air was similar to that of control flowers (Fig. 2). Treatment with STS and AOA also had only minor effects in delaying senescence (data not shown).

Response to α-amanitin treatment

Treatment of opening flowers with α-amanitin extended their longevity. In a typical experiment, flowers treated with the inhibitor were still open 24 h after the start of opening, whereas control flowers were already completely wilted (data not shown). To determine the critical time for the α-amanitin treatment, flowers were placed in solutions containing the inhibitor at 5 min intervals between 7 h and 8.5 h after stage 3. Treatment with the inhibitor before 7 h after stage 3 increased the life of all the flowers treated. Between 7 h and 8 h the response to α-amanitin treatment

Table 1. *Description of the primers used in the study*

Sequence no.	Direction	Primer
18S	Forward	5′-GAT TCT ATG GGT GGT GGT GCA T-3′
	Reverse	5′-CTC AAA CTT CCG TGG CCT AGA A-3′
F545	Forward	5′-CCA AAT CTT CGG AAA CAT CGT C-3′
	Reverse	5′-GGC AGG CTG ACA ACT ATC AGG T-3′
F935	Forward	5′-GAC CAG ATG TTC GAC GAG GAA A-3′
	Reverse	5′-TGC ACT TGC TTG ATC ATT GTG A-3′
F69	Forward	5′-TCT CAA CTC CAT TTC CCT CCA A-3′
	Reverse	5′-TGG AGG CTA TCT CAG TCG GTT C-3′
F453	Forward	5′-TTC TGG AAT GGC GAC AAT ACC T-3′
	Reverse	5′-CAT CAG CAT CCA CCA TCT TGA G −3′
R339	Forward	5′-GCT CGT TGG AAA TCG AAA CAG A-3′
	Reverse	5′-CCC AAG TAA GCC ATG TTT CCA C-3′
R261	Forward	5′-GAT TGC TGC TGA TGG ATC TGT G-3′
	Reverse	5′-GGC ATT GCT TTC AGC TTG TTC T-3′
R493	Forward	5′-ACC GCG ATT TGA TCC ACA TTA T-3′
	Reverse	5′-TCA GAA AGA AGA GCA GCA AGC A-3′
R709	Forward	5′-ACA GAC CCG TCT GAT GTG TTG A-3′
	Reverse	5′-GAC AAG CAA CTC AAG AGC AAC G -3′
R486	Forward	5′-TTG GAA TTG TCA CGA GGG AAG T-3′
	Reverse	5′-CAA ATC ACA ACC CGC ATC TAC A-3′

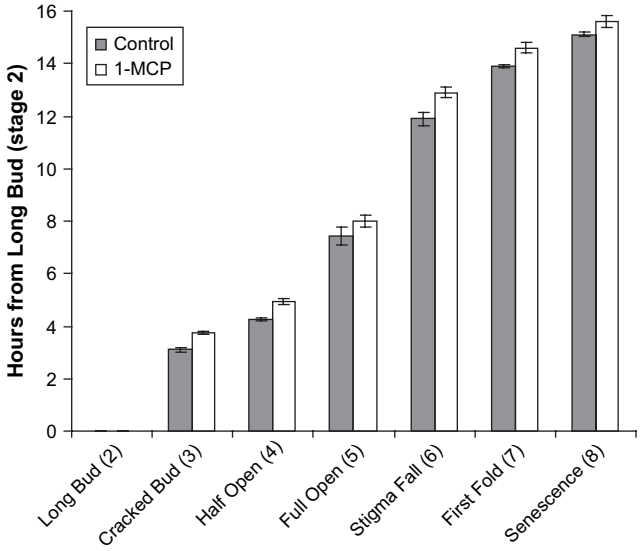

Fig. 2. Effect of 1-MCP on the life of *M. jalapa* flowers. Flowers were harvested at the long bud stage, placed in water, and then half were pretreated for 1 h with 200 nl l^{-1} 1-MCP. Time-lapse video images were recorded and used to assess the time taken to reach different stages during opening and senescence.

decreased linearly and thereafter the inhibitor had no effect in delaying senescence (Fig. 3).

Identification of genes differentially expressed during senescence

Corollas harvested 4 h and 9 h after opening were used to construct two subtractive cDNA libraries. The up-regulation library was enriched for genes expressed in 9-h-old corollas but not in 4-h-old ones. From this library, 889 clones

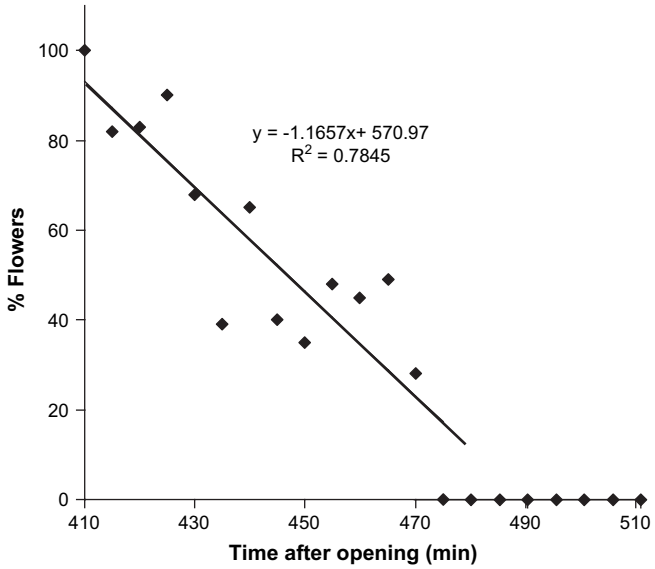

Fig. 3. Effect of α-amanitin on senescence of four o'clock corollas. At intervals after stage 3 (cracked bud), corollas were placed in a 20 μg ml^{-1} solution of α-amanitin. The progress of their senescence was recorded by time-lapse videography. The percentage of the flowers whose life was longer after the α-amanitin treatment was recorded.

were selected. The down-regulation library was enriched for genes expressed in 4-h-old corollas but not in 9-h-old ones. From this library, 1017 clones were selected.

One hundred and twenty-three of the clones from the up-regulation library and 140 clones from the down-regulation library were identified as being differentially expressed, based on a differential screen with cDNA isolated from 4-h-old and 9-h-old corollas. These clones were sequenced and assembled into 82 and 65 contigs, respectively, then compared with sequences in the GENBANK databases. The sequences had diverse putative functions which are summarized in Tables 2 and 3.

Genes encoding several types of DNA-binding proteins were prominent in the up-regulation library, including a bZIP transcription factor, and a homeodomain-leucine zipper (HD-Zip) transcription factor (Table 2), which was moderately abundant, since five of the selected cDNAs represented this sequence. The most abundant sequence was a petunia NEC1 homologue represented by 16 clones (Table 2). The second most abundant sequence, represented by six clones, encoded a homologue to the tomato predominantly leaf-expressed protein with no reported function (Table 2). Other moderately represented genes in the up-regulation library included a seed imbibition protein, an ankyrin repeat family protein, a dynein light chain type 1 protein and several sequences with unknown functions (Table 2). Genes potentially involved in protein turnover in the up-regulation library included several polyubiquitin genes, a gene encoding an aspartic proteinase, and a gene encoding a RING zinc finger ankyrin protein (Table 2). Several defence/stress/senescence related

Table 2. *Putative functions of genes identified in the up-regulation library*

Potential role	Number	Examples
DNA binding/ transcription	4	bZIP transcription factor; homeobox-leucine zipper protein; BTB/POZ domain-containing protein, jumonji (jmjC) domain-containing protein
Signalling	4	C2 domain-containing protein; calmodulin-binding protein-related; protein kinases; putative acid phosphatase; phosphoribulokinase/uridine kinase
Transport	2	Proton-dependent oligopeptide transport (POT) family protein; peptide transporter
Protein degradation	4	Polyubiquitin; aspartyl proteases; RING zinc finger ankyrin protein
Amino acid metabolism	5	Branched-chain amino acid aminotransferase; putative 2-isopropylmalate synthase; dihydroorotate dehydrogenase
Carbohydrate metabolism	5	Seed imbibition protein; starch branching enzyme class II
Cell wall-related	4	Cinnamoyl CoA reductase; cinnamyl-alcohol dehydrogenase; invertase/pectin methylesterase inhibitors; beta-1,2-xylosyltransferase
Defence/stress/ senescence-related	6	Pathogenesis-related thaumatin; polygalacturonase inhibiting protein; senescence-related protein; dehydration-responsive protein-related
Cell division-related	3	Putative RNA-binding protein MEI2; meiosis protein-related; putative growth regulator protein
Ethylene biosynthesis	1	1-Aminocyclopropane 1-carboxylate (ACC) synthase
Other	17	Petunia NEC1; *Lycopersicon esculentum* (=*Solanum lycopersicum*) predominantly leaf-expressed protein; *Arabidopsis thaliana* dynein light chain type 1
Unknown/no match	27	

Table 3. *Putative functions of genes identified in the down-regulation library*

Potential role	Number	Examples
DNA binding/ transcription	2	Circadian clock-associated 1 (CCA1); MYB transcription factor
Signalling	3	39 kDa EF-hand protein; protein kinases; shaggy-related protein kinase gamma
Transport	3	ABC transporters; water channel protein
Protein degradation	3	Aspartyl proteases; ATP-dependent Clp protease proteolytic subunit; zinc finger (C3HC4-type RING finger) protein
Phytochrome A-related	2	FIN219; phytochrome A-related
Lipid metabolism	2	Lipid transfer protein; GDSL-lipase
Cell wall-related	3	UDP-glucoronosyl/UDP-glucosyl transferase protein
Defence/stress/ senescence-related	4	Phosphonate biosynthesis related; HMGR CoA reductase; drought-induced protein
Secondary product synthesis	3	Terpene synthase/cyclase; alcohol acyltransferase
Aux/IAA	2	Aux/IAA2; auxin-responsive protein IAA1
Other	13	Putative male sterility 2 protein; nitrogen regulation protein; cytochrome P450 monooxygenase; short-chain type alcohol dehydrogenase
Unknown/no match	25	

genes were also present, including an ACC synthase homologue, a pathogenesis-related thaumatin family protein, and a dehydration-responsive protein. In addition, this library contained homologues of genes related to cell division, including an RNA-binding protein MEI2 and a meiosis-related protein.

In the down-regulation library (Table 3) two Myb transcription factor homologues were identified, one being a *Mesembryanthemum crystallinum* circadian clock-associated (CCA1) homologue. Genes encoding proteins associated with the phytochrome A pathway were identified; one was a *FIN*219 homologue, the other a WD-40 protein homologue. Down-regulated genes encoding proteins involved in signalling included an EF-hand protein gene and two protein kinases (Table 3). A sequence encoding an aspartyl protease was highly represented in the down-regulation library. Other sequences encoding proteins involved in protein turnover included an ATP-dependent Clp protease proteolytic subunit and a gene containing a RING domain (Table 3). Genes encoding a short-chain alcohol dehydrogenase and a terpene synthase/cyclase were also highly represented in the library.

Quantitative analysis of the expression of genes during flower development and senescence

As determined by quantitative real-time PCR analysis, transcript abundance of most of the up-regulated genes increased dramatically (Fig. 4) between stages 5 and 6 (between 4 h and 11 h after flower opening). The gene encoding the RING zinc finger ankyrin protein showed the highest change among the selected up-regulated genes (Fig. 4A), increasing 40 000-fold from stage 5 to stage 8. At stage 8, transcript abundance was similar to that of the abundance of the highly expressed 18S internal control (data not shown). Expression of the HD-Zip gene (Fig. 4B) began to increase at stage 4 (half open) and reached a plateau at stage 6 (stigma fall), when its abundance was >200 times that in long buds. Transcripts of the gene encoding a tomato predominantly leaf-expressed protein homologue also showed a large increase, about 600-fold, from stage 5 (fully open) to stage 6 (stigma fall) (Fig. 4C). Expression of the BTB/POZ domain-containing sequence (Fig. 4D) increased 50-fold between stage 5 and stage 6 and continued to increase, to 160-fold, by stage 8 (senesced). Expression of the R29 senescence-associated gene (Fig. 4E) and of the ACC synthase homologue (Fig. 4F) showed more complex

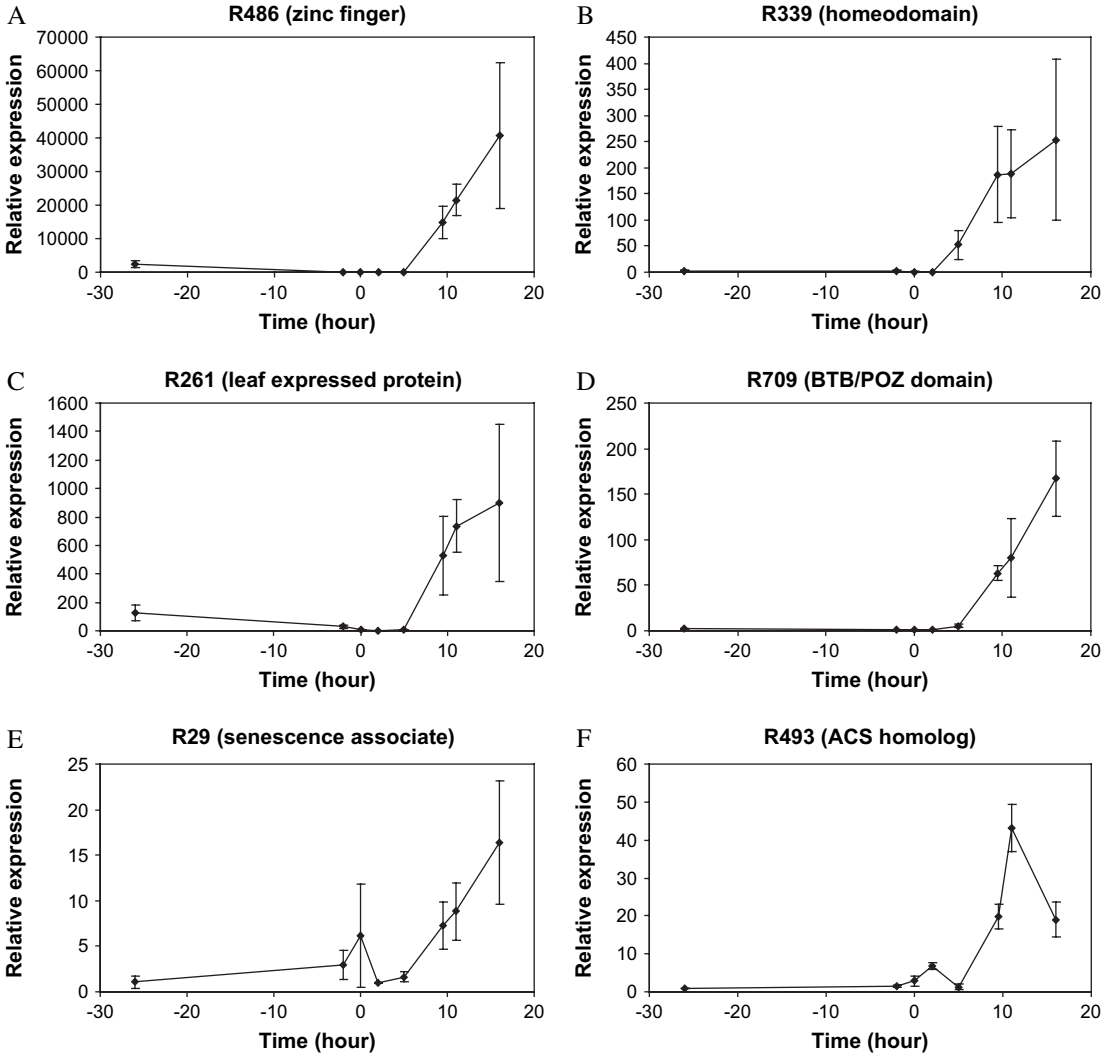

Fig. 4. Expression patterns of selected genes from the up-regulation library. Abundances were determined by comparison with an internal 18S control, and were calculated relative to the lowest abundance, which was given a value of 1. Putative gene functions are noted in parentheses.

patterns; in the case of ACC synthase, a small peak at stage 4 (half open), and a much larger peak, 45 times the initial level, at stage 6 (stigma fall).

All the down-regulated genes had their highest expression levels at or before the flowers were fully open (stage 5), then fell to low levels before or early in senescence (Fig. 5). Expression of the CCA1 homologue peaked at stage 4 (half open) with a 250-fold increase over the expression at stage 1 (small bud) (Fig. 5A). The other Myb transcription factor was most highly expressed at stage 2 (long bud), and then decreased (Fig. 5B). The aspartyl protease highly represented in the down-regulation library had the most dramatic change among the selected down-regulated genes. It increased over 2000 times from stage 2 (long bud) to stage 3 (half open), but had fallen to its initial level by stage 6 (Fig. 5C). For the EF-hand protein homologue, there was also a sharp increase in transcript abundance between stage 2 (long bud) and stage 3 (cracked bud), and abundance fell to the levels present in small bud (stage 1) by the onset of senescence (Fig. 5D).

Discussion

The rapid opening and short life of the flowers of *Mirabilis jalapa* make this species an interesting model for studying the control of petal senescence. In other ephemeral flowers, control of senescence has been shown to be ethylene sensitive (morning glory, hibiscus) or ethylene independent (daylily, iris) (Woltering and van Doorn, 1988). *Mirabilis jalapa* flowers show a mixed pattern of senescence, in which treatment with ethylene accelerates senescence, and inhibition of ethylene action has little effect. Unlike the 'intermediate' senescence pattern in daffodil (Hunter *et al*., 2002), pollination of *M. jalapa* flowers does not alter the pattern of senescence

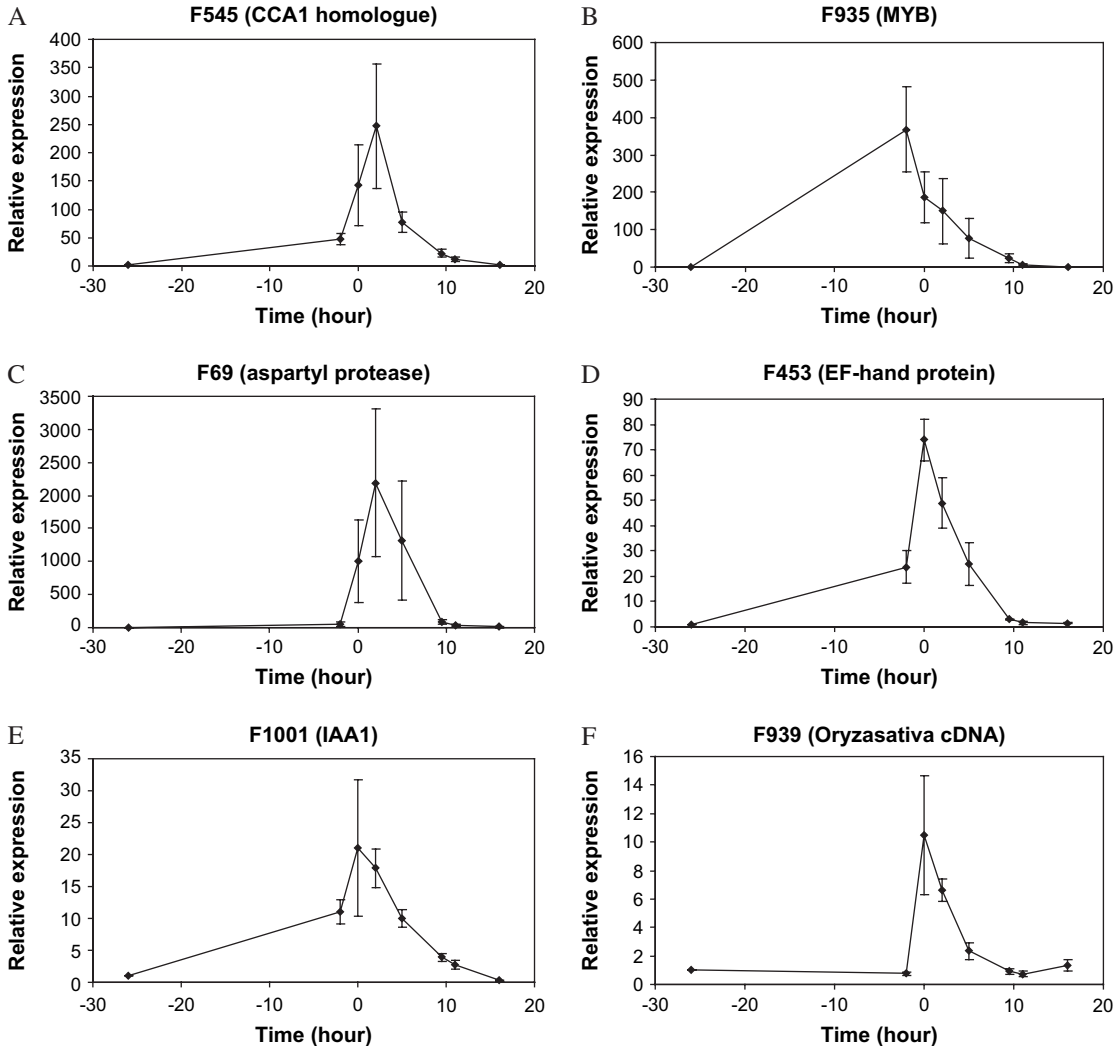

Fig. 5. Expression patterns of selected genes from the down-regulation library. Abundances were determined by comparison with an internal 18S control, and were calculated relative to the lowest abundance, which was given a value of 1. Putative gene functions are noted in parentheses.

(Li *et al*., 1994). Indeed, one of the attractive features of this flower as a model system is that the detached corolla senesces in exactly the same fashion as the intact flower.

The acceleration of senescence seen in *M. jalapa* flowers treated with ethylene is particularly interesting, in that it is clearly due largely to a dramatic reduction (from 4 h to 2 h) in the time taken to progress from full opening to stigma fall (the first sign of senescence) (Fig. 1). Ethylene-treated flowers open at the same time as control flowers, and after stigma fall the progression to complete senescence is independent of the presence of ethylene. In some way, therefore, this hormone accelerates the triggering, but not the execution of the senescence process.

Treatment at different times with α-amanitin, a classic inhibitor of eukaryotic DNA-dependent RNA synthesis (Lindell *et al*., 1970), demonstrated the importance of the period between full opening and stigma fall. The inhibitor extended the life of all the flowers treated <7 h after the start of opening, and had no effect on any flowers treated after 8 h. It was concluded from these data that transcripts synthesized during this period determine the timing of senescence. The differential screening therefore examined genes that were differentially expressed prior to (4 h) and after (9 h) this period, in the expectation that the enriched libraries would include the key genes involved in the inhibition or initiation of senescence. The resulting libraries included several transcription factors and other intriguing genes that provide leads for further research on the initiation and execution of floral senescence.

The opening and senescence of *M. jalapa* flowers appears to be under photoperiodic control – if plants are transferred to a growth chamber with a reversed photoperiod, floral opening and senescence is synchronized to the new photoperiod within 2 d (X Xu *et al*., unpublished results). It was therefore interesting that transcripts of CCA1, a Myb transcription factor that has been reported

to play a key role in the photoperiodic clock, showed a striking peak in abundance during flower opening and maturation (Fig. 5A). Interestingly, this pattern was not present in younger buds, suggesting that the expression of this gene was also controlled by a developmental signal. Two other sequences related to light sensing, one showing homology to the *Arabidopsis* FIN219 and the other to a phytochrome A-related WD-40 repeat family protein (At3g15354), were also differentially expressed in *M. jalapa* flowers. These data are consistent with a model where the opening and senescence of *M. jalapa* flowers is under the control of the circadian clock, itself entrained by light input sensed by the phytochrome A pathway (McClung, 2001). All of the down-regulated genes identified by differential expression showed a peak in abundance during flower opening, and the clock-related genes are no exception. Another Myb gene homologue (F935) was induced very early in flower opening—its transcript abundance was highest in long buds (Fig. 5B). It remains to be established whether these genes are solely involved in directing floral opening and expansion, or whether reduction in their abundance permits the onset of flower senescence.

Identifying factors controlling floral senescence was a major goal of this study and it had been anticipated that changes in abundance of a range of regulatory genes, particularly transcription factors would be seen. While Myb transcription factors were identified as down-regulated genes, probably playing a role in the control of flower opening, the only transcription factors identified in the up-regulated library were homologues of bZIP and HD-Zip proteins. HD-Zip proteins are transcription factors unique to plants and they have been grouped into four families (Sessa *et al*., 1994). The HD-Zip transcription factor isolated in the present study belongs to the HD-Zip I family, a family that, in *A. thaliana,* includes *Athb-7* and *Athb-12* (Sessa *et al*., 1994; Lee and Chun, 1998). These genes are induced by water stress and abscisic acid (ABA) treatment (Söderman *et al*., 1996; Lee and Chun, 1998), and it seems possible that the *M. jalapa* genes may be induced in response to the changing osmotic and water relations of the opening and senescing flower.

Flower senescence has been characterized as an example of programmed cell death (PCD) in plants. PCD in plants is thought to be homologous to the process that is so well described in animal systems, invoked by the activity of specific proteases, possibly triggered by changes in mitochondrial structure, and involving an elaborate cascade of catabolic processes leading to degradation of macromolecules and, ultimately, death of the cell (Trobacher *et al*., 2006). In other species, genes encoding cysteine proteases have been shown to be induced during the onset of senescence (Jones *et al*., 1995; Panavas *et al*., 1999; Rubinstein, 2000; Hunter *et al*., 2002; Breeze *et al*., 2004). Although no cysteine protease was found in our libraries, the genes that were isolated are consistent with the notion that protein degradation is an important component of the senescence programme. An early event was the up-regulation of putative aspartyl proteinases. Most plant aspartyl proteinases belong to the A1 family (Simões and Faro, 2004), which has 59 members in the *Arabidopsis* genome (Beers *et al*., 2004). It has been suggested that the aspartyl proteinases may be involved in diverse functions, including protein processing and/or degradation, sexual reproduction, stress responses, and senescence/PCD (reviewed in Simões and Faro, 2004). Aspartyl proteinases were also found to be up-regulated in senescing petals of daylily and *Alstroemeria* (Panavas *et al*., 1999; Breeze *et al*., 2004). In the present study, expression of one of the isolated aspartic proteinases increased 2000-fold from stage 2 (long bud) to stage 4 (half open) and then decreased to the basal level at stage 6 (stigma fall).

Regulation of growth and development by controlled protein turnover frequently employs the ubiquitin pathway, in which proteins are selected for degradation, linked to ubiquitin, then degraded in the proteasome (Finley and Chau, 1991; Ciechanover and Schwartz, 1998). It has been suggested that the ubiquitin pathway is involved in the degradation of petal proteins during floral development and senescence in daylily (Courtney *et al*., 1994), and a proteasome inhibitor, Z-leu-leu-Nva-H, was shown to delay senescence in iris tepals (Pak and van Doorn, 2005). Our up-regulated library contained a gene encoding polyubiquitin, an essential element in the ubiquitin pathway. In addition, abundance of transcripts of a gene encoding a RING zinc finger ankyrin protein increased 40 000-fold as the flowers senesced (Fig. 4). At stage 8, its transcripts were nearly as abundant as those of the highly expressed 18S transcript that were used as a real time PCR control. The sequence of this gene was highly homologous to that of E3-type binding proteins, which are ubiquitin ligases (Wang *et al*., 2006). These proteins target proteins for proteolysis and are usually thought to be responsible for the specificity of the ubiquitin pathway (Schell and Hicke, 2003). Many RING domain-containing proteins have E3 activity (Stone *et al*., 2005), including the *Arabidopsis XB3* homologue *XBAT32* (Nodzon *et al*., 2004). There were five *XB3* homologues in *Arabidopsis*, *XBAT31* to *XBAT35* (Nodzon *et al*., 2004). The gene isolated in the present study was most similar to *XBAT31* which has no identified function. *XBAT32* has been shown to regulate lateral root development in *Arabidopsis* (Nodzon *et al*., 2004). Cortical parenchyma cells adjacent to the lateral root emergence site were specifically degraded (Bonnett, 1969), and this PCD did not occur in a null *xbat32* mutant (Kosslak *et al*., 1997). Since related genes are closely associated with PCD events in other systems, it seems very interesting to pursue the hypothesis that the *M. jalapa* RING zinc finger protein, that shows such dramatic up-regulation during senescence, may play a key role in the control of the process.

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